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A PHYSIOLOGICAL TEST FOR THE ACTIVITY OF VITAMINE PREPARATIONS.

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Progress in the chemical isolation of vitamine depends largely upon the success of the physiological tests available for guiding the fractionations. Any improvement in the accuracy and rapidity of these tests is undoubtedly a matter of importance. The technique which has gradually been developed in the Hygienic Laboratory is believed to possess advantages which may be appreciated by others engaged in experiments on the isolation of vitamine.

The effect produced on fowls by vitamine deficiency was early utilized for studies of both the distribution of the antineuritic vitamine in natural foodstuffs and in fractions obtained by chemical operations. At first the curative effect of vitamine concentrates upon fowls, brought to the polyneuritic state by vitamine deficiency, was utilized as a test for the activity of the unknown sample. This has gradually been supplanted by a preventive method, which consists in adding measured amounts of the vitamine material to a vitamine-deficient diet and noting the amount required to replace the deficiency. This procedure has the advantage that birds in a normal condition are used, rather than those in an abnormal or what may, perhaps, be more properly described as a state of unstable nutritional equilibrium.

The most suitable bird to use for the tests is the common pigeon, and a satisfactory vitamine-deficient diet is polished rice. Upon such a diet pigeons soon begin to lose weight, and after about three weeks decline to some 60 to 70 per cent of their original weight and exhibit characteristic symptoms of polyneuritis. If, however, samples containing the antineuritic vitamine are administered daily or on alternate days to such pigeons, fed only on rice, no apparent change in their condition takes place. The presence or absence of vitamine will be indicated by comparing the weight curves of the birds receiving the vitamine with those receiving none—that is, with the control birds. In the past, whenever one or more samples were to be tested for their vitamine content, a group of pigeons was placed on a rice diet and measured doses of the samples were given to some of the birds, the others being retained as controls. At the end of the experiment, all of the birds were given a mixed diet to restore them to their initial condition. They could then be again used for a similar experiment if desired.

Considerable experience has shown that the tests can be conducted much more satisfactorily if all the birds on a rice diet are given "activated solid"[1] on alternate days, in doses just sufficient to prevent an appreciable loss in weight. They thus become accustomed to the rice diet and reach a state approaching vitamine equilibrium. If now the doses of "activated solid" are replaced by those of the samples to be tested for their vitamine content, there will be a very quick response to any diminution of vitamine, and thus a very prompt indication of the character of the sample in question. The administration of "activated solid" can then be resumed, and the birds made ready for a second test within another 10 days or so. Proceeding in this manner a single group of pigeons can be used for periods of many months, and for testing large numbers of different samples. The group in use at this laboratory has now been on an exclusive diet of polished rice (with addition of active vitamine preparations) for more than six months.

The practical consideration underlying the adoption of the above plan was the observation that, whenever pigeons are put upon polished rice, some time is required for them to become adjusted to the new diet. When they receive polished rice for the first time they may eat excessively and show abnormal weight changes. When they are placed on this diet a second time, after a recuperation period on mixed grains, they refuse to eat until driven to it by hunger, and thus again exhibit abnormal weight changes. If kept on rice constantly, they appear to standardize their eating habits, and irregularities in the weight curves are greatly diminished.

The question whether rice plus fuller's earth containing adsorbed vitamine is an adequate diet for pigeons, has not been taken into consideration in the present test. Although the birds do not mate and reproduce, they show no perceptible signs of malnutrition for relatively long periods and are constantly in a state in which the withdrawal of vitamine is manifest by prompt and definite effects. It is believed that an attempt to modify the diet by addition of constituents intended to improve its quality might have the effect of diminishing the accuracy of the test.

In a practical way it has been found convenient to keep groups of 10 or more pigeons in compartments of some 300 cubic feet in volume. A bottle with wide mouth, clamped in an inverted position over a small pan, makes a satisfactory receptacle for the rice supply. A fairly large tank through which a slow stream of water flows should, if possible, be provided. A thin layer of coarse sand is spread over the floor of the compartment and should be renewed frequently.

Each bird is provided with a numbered leg band and is weighed on each alternate day, and immediately after weighing it is given, by mouth, the "activated solid" or sample being tested. On plotting the series of weighings, a clear picture is obtained of any change which may take place in the condition of the bird.

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[1] See Improved Method of Preparing Vitamine-Activated Fuller's Earth. By A. Seidell. Public Health Reports, *37*, 801 (Apr. 7, 1922). Reprint No. 738.

The vitamine samples are most conveniently administered in gelatine capsules, each being moistened with water immediately before being given to the pigeon. For this reason it has been found advantageous to convert all samples of vitamine to their adsorption combination with fuller's earth. The dry powder thus obtained can be readily measured in capsules of standard size instead of being weighed. This greatly simplifies the preparation of the capsules for dosage, and provides against deterioration or change in the activity of the vitamine during the period of the test.

The procedure for preparing the vitamine-fuller's earth adsorption combination for physiological tests consists in dissolving the weighed sample in about 500 c. c. of water, or diluting a solution containing a known amount of vitamine solids to about this volume, and adding such an amount of fuller's earth that each 0.1 gm. corresponds to 1, 2, or more milligrams of the vitamine solids present. The mixture is actively shaken during one-half hour or longer, filtered on a Buchner funnel, and washed with water, then alcohol, and finally with ether to facilitate rapid drying. The dried sample is then measured into gelatine capsules, each containing 0.1 gm. or more, as the case might be, and these capsules are given to the pigeons after each weighing as described above. In this way all conditions of the test are kept uniform and the only variable is the antineuritic activity of the unknown sample.

In regard to the delicacy of the test, it has been found that unmistakable differences in the weight changes of the pigeons can be detected for quantities of sample, varying by one or two tenths of a gram. Thus, for example, pigeons receiving 0.3 gm. doses of a fuller's earth-vitamine combination, corresponding to 3 milligrams of the unknown sample, have been found to remain stationary in weight, while those receiving 0.2 gm. doses, corresponding to 2 milligrams, declined noticeably, and those receiving 0.1 gm. showed a more rapid decline. Differences which have been obtained between the effects of 4 and 6 milligrams of adsorbed vitamine solids and between 7.5 and 10 milligrams are other examples of the delicacy of the method.

It has been found that the time required for detecting the differences, resulting from variation in dosage, is shorter than before adoption of the technique here described. Positive results can usually be obtained within two weeks, and frequently within a shorter time. Samples devoid of vitamine are identified most promptly.

There is, of course, a necessity for caution against giving doses in excess of the amount required for maintenance of weight. Such quantities are not indicated by the test, and the best practice requires that for each sample being tested at least some of the pigeons receive slightly deficient doses. The matter of the selection of the dosage of unknown samples is, of course, the principal difficulty, and accurate results require repetition of the test with repeated adjustments of the dosage. In general, each sample should be tested in two or more different dosages and not less than three pigeons should be used for each dosage.

Although the method described above has been developed particularly for controlling the fractionation steps in the attempts to isolate vitamine from brewer's yeast, it can be used equally well for testing the antineuritic vitamine content of a great variety of vitamine preparations. In such cases, the vitamine portion of the sample should be obtained in the form of an aqueous solution (by any appropriate means), and this solution should be agitated with fuller's earth. The vitamine-fuller's earth adsorption combination is then removed and obtained in the form of dry powder, as already described. The protective effect of this product, as compared with that of standardized "activated solid," will give a satisfactory estimate of the vitamine content of the unknown material.

In the course of experiments made on the isolation of vitamine from brewer's yeast, some evidence was obtained as to the approximate weight of active material required to replace the deficiency of a polished rice diet for pigeons. This evidence was based upon a comparison of the milligrams of nitrogen (determined by the Kjeldahl method) present in samples of fuller's earth containing adsorbed antineuritic material responsible for the protective action. It is provisionally assumed that the pure antineuritic vitamine actually contains nitrogen, although it should be stated that this assumption is not proved, as pure vitamine has not been isolated.

Experiments have shown that a sample of the new "activated solid,"[1] which contains 1.5 per cent of nitrogen protects pigeons on a rice diet in doses of 0.1 gram given on alternate days. On the nitrogen basis, the protective dose is, therefore, 1.5 milligrams. Using "activated solid" prepared by the old method and containing 2 per cent nitrogen, the average protective dose was at least 0.15 gram, which, on the nitrogen basis, corresponds to 3 milligrams. It is, therefore, apparent that at least one-half of the nitrogen in the old sample is not present as vitamine nitrogen.

In regard to what proportion of nitrogen in the "activated solid" prepared by the new method is present in vitamine combination, some light is obtained from the following experiment. The extracted material, removed from the sample of the new "activated solid" by barium hydroxide extraction, was readsorbed by being agitated with a fresh portion of fuller's earth. Tests of this material, which contained only 0.17 per cent of nitrogen, showed that protection was afforded by doses of 0.3 gram. This, on the nitrogen basis, corresponds to 0.51 milligram instead of 1.5 milligrams nitrogen found for the original "activated solid," and, therefore, indicates that not more than one-third of the nitrogen in the "activated solid" prepared by the new method is present in vitamine combination.

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[1] Seidell. Loc. cit.

In view of this result, it appeared of interest to ascertain what is the lowest obtainable amount of nitrogen capable of replacing the vitamine deficiency of a polished rice diet for pigeons. Nitrogen was, therefore, determined in those fuller's earth preparations which had been found, by comparative tests, to protect pigeons in doses corresponding to the smallest amounts of active material. Of these the most favorable sample was one which had been made by the fuller's earth adsorption of the highly active silver-free residue obtained from an ammoniacal silver nitrate precipitate. It contained 0.08 per cent of nitrogen, determined by the Kjeldahl method, and protected in doses of 0.1 gram given on alternate days. Therefore, in this case the deficiency of the rice diet was replaced by doses of approximately 0.08 milligram of nitrogen in vitamine combination given on alternate days, which is equivalent to 0.04 milligram daily.

On the basis of estimations of nitrogen in samples of highly active vitamine fractions obtained from silver precipitates, and after deducting the nitrogen present in these residues as nitric acid, it is probable that the free vitamine base contains somewhat more than 16 per cent of nitrogen. If it is assumed that 20 per cent is present, the 0.04 milligram of active nitrogen, referred to in the preceding paragraph, corresponds to 0.2 milligram of uncombined vitamine base.

Although this figure is simply a rough approximation and may be somewhat too high, it serves to convey an idea as to the quantity of vitamine required for normal nutrition. It is useful in a practical way in work on the isolation of vitamine, in showing the order of magnitude of dosage which must be approached with fractions believed to be pure or nearly pure vitamine. With this figure in mind, it certainly will not be necessary to undertake identification tests or analyses upon fractions which, in daily doses greater than one-fourth milligram, do not protect pigeons from loss in weight on polished rice.